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The effects of elevated CO_2 and nitrogen availability supersedes the need for nodulation in peas grown under controlled environmental conditions

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The effect of elevated CO_2 (1 000µmol mol^{-1}) on the growth and leaf development of nodulating and non-nodulating *Pisum sativum* L. var. Greenfeast was studied under both nitrogen (6mM) and nitrogen-free nutrient solution supply. Plants were sown and kept under ambient and elevated CO_2 throughout their growth period. Studies were based on the use of the plastochron index as a measure of plant growth. Results show that short-term exposure to elevated CO_2 induced plant growth, irrespective of treatment. Long-term elevated CO_2 treatment did not affect rate of leaf appearance (RLA) in nodulated plants irrespective of mineral N supply but induced RLA in non-nodulating plants supplied with mineral N. Supplied N induced leaflet elongation rate significantly under both ambient and elevated CO_2 in nodulated and non-nodulated plants. Leaflet elongation rate was not significantly affected by nodulation but was increased by high CO_2. The results reported here, show that a CO_2 level of as high as 1 000µmol mol^{-1} may not significantly affect the growth of nodulating *P. sativum* L. Our data suggest that elevated CO_2 will enhance canopy size, provided adequate soil N is available. In addition, canopy size will be significantly increased under elevated CO_2 in non-nodulating plants, provided adequate soil N is applied.

Abbreviations: E = elevated CO_2, A = ambient CO_2, PI = plant plastochron index, R = with nodules/Rhizobium, r = without nodules, N = with nitrogen supply, n = no nitrogen supply, RLA = Rate of leaf appearance, L_fER = leaflet elongation rate, DAG = days after germination

Introduction

Interactive effects of elevated CO_2 and other environmental factors on plants remains a subject of much discussion. Though some workers have proposed that high [CO_2] effects on plant growth were not affected by environmental stress factors (Idso and Idso 1994), others have reported or concluded that high [CO_2] effects vary among plant species grown under different environmental conditions (Kimball 1983, Poorter 1993, 1998, Thompson and Woodward 1994, Hunt *et al.* 1995, Ziska *et al.* 1996, Brunce 1998, Wu and Wang 2000). According to Kramer (1981) and Poorter (1998), the positive effects of CO_2 cannot be mentioned or inferred when other environmental factors are limiting. Daepp *et al.* (2001) have reported that high growth rate under high nutrients availability promotes a strong response to elevated CO_2. Legumes with their unlimited access to the N_2 pool in the atmosphere via symbiotic fixation, usually show a stronger response to elevated CO_2 than non-fixing plant species (Zanetti *et al.* 1997). However, strong positive effects of CO_2 on legumes relative to effects on non-legume species were obtained with high mineral nutrient supply (Nijs *et al.* 1989, Körner 1995, Pritchard *et al.* 1997).

Philips *et al.* (1976) have demonstrated that short-term increases in CO_2 levels promote N_2 reduction in peas (*Pisum sativum* L.) by enhancing root nodule functioning, whereas long-term CO_2 enrichment promotes N_2 reduction by increasing plant and root nodule development (due to increase in root area available for nodule formation) which result from excess nitrogen fixed during early period of treatment. However, increased nodulation is not maintained during long-term exposure in the presence of supply of mineral nitrogen as further nodule initiations are probably inhibited in such treatments. Rivière-Rolland *et al.* (1996) showed that decrease in Rubisco activity occur in peas grown in elevated CO_2 and nitrogen-limiting condition. A threshold value for the N status according to Rivière-Rolland *et al.* (1996) occurs above which Rubisco is not down-regulated by high CO_2.

According to Reddy *et al.* (1998), the effects of increased [CO_2] on growth is primarily manifest due to changes in leaf area production with smaller effects of increased [CO_2] on photosynthetic rate, nitrogen and water use efficiency. Under elevated CO_2, leaf area responses may be limited by deficits of nutrients or water (Reddy *et al.* 1998). Studies on leaf development need to encompass analyses of the leaf appearance and expansion/elongation rates which according to Reddy *et al.* (1993), are factors involved in determining leaf area changes. Plastochron index (PI) may be used in determining plant and leaf morphological ages; periodic

leaf length measurements used in plastochron index analysis can be used in determining leaf elongation rate, while plastochron duration is the rate of leaf appearance. Thus, plastochron index analyses would help in the complete study of leaf development under elevated CO_2.

The effects of long-term exposure of pea plants to elevated CO_2, nodulation status and the influence of nitrogen availability on these factors, prompted us to undertake studies of plant growth under controlled environmental conditions. The aim of the research reported here was to determine the influence of [CO_2], N availability, and nodulation on rate of appearance and elongation of leaves under the conditions described here. Controlled growth conditions allow studies of potential up-regulation of plant growth where changes in [CO_2], [N] and nodulation status are implemented. We contend that the use of the plastochron index and plastochron duration are excellent measures for determining relative plant growth rate on a per unit time basis, under these experimental conditions.

Materials and Methods

Plant culture

Sterilised (3.5% m/v sodium hypochlorite solution to prevent nodulation; Rivière-Rolland *et al.* 1996) and non-sterilized seeds of *P. sativum* var. Greenfeast were sown in sterilized and non-sterilized potting soil respectively (Greenfingers, South-Africa). Upon germination, six seedlings were transplanted per pot. Five grams of slow-releasing fertilizer (NPK 2:3:2; Wonder Horticultural Products, Johannesburg, South Africa) was added to the soil in pots (185mm x 185mm, 165mm deep) prior to transplanting seedlings. Twelve plants were used per treatment. Pots were irrigated with either full strength complete (with 6mM nitrogen) or nitrogen-free Long Ashton nutrient solution (Hewitt 1966) depending on treatment group.

Plant growth parameters

Treatments were based on six parameters: elevated CO_2 (E, 1 000µmol mol^{-1}) and ambient CO_2 (A, 360µmol mol^{-1}); non-nodulating (r) and nodulating (R); irrigation with nutrient solution containing mineral N (N) and irrigation with nitrogen-free nutrient solution (n). Pots were assigned symbols rEN and rAN denoting non-nodulating/non-nitrogen-fixing plants supplied with N+ nutrient solution under elevated and ambient CO_2 respectively; REn and RAn denoting nodulating/nitrogen-fixing plants supplied with N-free nutrient solution under elevated and ambient CO_2 respectively while REN and RAN stands for nodulating/nitrogen-fixing plants supplied with N+ nutrient solution. The group, non-nodulating supplied with N-free nutrient solution were left out of the experiments because preliminary tests under both controlled and greenhouse conditions show that without nitrogen supply, non-nodulating pea seedlings die within two weeks after germination. Preliminary experiment by Arnone and Gordon (1990) also demonstrated that in un-inoculated red alder, plants receiving nutrient solution without nitrogen do not grow. Two pots with six plants were used for each treatment. Pots were placed in a randomised blocked matrix design and positions were changed daily in a definite pattern to prevent chamber effect.

Growth conditions

Plants were grown in two growth chambers (Conviron Model S10H, Controlled Environments Ltd, Winnipeg, Canada) under 25°C/18°C day/night conditions and with a 16h photoperiod. CO_2 concentration was maintained at 360 and 1 000µmol mol^{-1} respectively with insignificant fluctuations within ±15µmol mol^{-1} of the [CO_2] set point. CO_2 was monitored using the on-board computer-controlled CO_2 analysers. Plants were illuminated using a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, USA) and frosted incandescent 60W bulbs (Philips, Eindhoven, The Netherlands). Photosynthetically active radiation (PAR, 400–700nm) was approximately 250µmol m^{-2} s^{-1} at 20cm above soil level as measured with a Li-85A Quantum sensor (Li-Cor Inc, Nebraska, USA) as recommended by Olivier and Annandale (1998).

Leaflet measurement

The first true leaf of the pea plant (with oval-shaped leaflets and tendrils) is borne on node 3. Node numbering is taken from the base starting with zero. Leaflet length measurements were recorded from the first pair of leaflets on node 5. Measurements were made using an electronic digital calliper at the same time each day, throughout the vegetative growth stage.

Plastochron analysis

The average leaflet lengths were recorded for each node on the plants from 11 DAG to 20 DAG since plastochron analysis is limited to period of active vegetative growth. Plastochron index of plants (PI) per day was calculated for each plant based on the formula:

$$PI = n + \frac{\log L_n - \log 20}{\log L_n - \log L_{n+1}}$$

where n is the serial number (counting from the shoot base) of that leaf which just exceeds 20mm; log L_n is the natural logarithm of the length of the leaf n; and log L_{n+1} is the natural logarithm of the next or subsequent leaf with a length that is less than 20mm (Ade-Ademilua and Botha 2005).

Statistical analysis

Unless otherwise stated, the average leaflet lengths were recorded for each node and 12 replicates were used for the analysis. Data were analysed for each plant. Descriptive and regression analyses were carried out using Excel 2000. One-way analysis of variance (ANOVA) was carried out at 0.1%, 1% and 5% levels of significance.

Results

The growth of *Pisum sativum* L. var. Greenfeast under elevated (E) CO_2 (1 000µmol mol^{-1}) in comparison to ambient (A) CO_2 (360µmol mol^{-1}) was studied in different groups of

experimental plants:

1. Nodulated plants (R) supplied with nitrogen-free nutrient solutions (n)
2. Nodulated plants (R) supplied with nutrient solution containing mineral nitrogen (N) and,
3. Non-nodulated plants (r) supplied with nutrient solution containing mineral nitrogen (N)

Under controlled environment, seeds germinated within two days after sowing in all treatments. Leaflet elongation was rapid (exponential phase) for the first five days after leaf unfolding. The rate of increase in leaflet elongation was therefore determined using values within the period of exponential growth, that is five days.

Effect of elevated CO_2 on nodulating plants without mineral N supply

The average plastochron index (PI) of nodulated plants supplied with N-free nutrient solution under elevated (REn) was significantly higher than that of their counterparts under ambient CO_2 (RAn), throughout the experimental period as denoted in Figure 1A (P = 0.01 at 11 DAG and P = 0.001 from 12–20 DAG). The plastochron duration (inverse of slope) of REn-treated plants was shorter (2.64 ± 0.06 days) than that of RAn-treated plants (2.74 ± 0.12 days) but the values were not significantly different at P = 0.05. This suggests that a positive response to elevated CO_2 by nodulated plants occurs during the very early stages of growth and this effect had put these plants ahead (in terms of plastochron age) of their counterparts grown under ambient CO_2. Figure 1B shows that leaflets of REn-treated plants grew faster (3.58 ± 0.39mm day^{-1}) than those of RAn plants (3.01 ± 0.16), but the difference was also not significant at P = 0.05. Long-term exposure of nodulated *P. sativum* L. to elevated CO_2 does not seem to influence the growth of leaves positively. Early chlorosis was noticed on leaves of REn-treated plants by 21 DAG as against that associated with end of plant life cycle in other treatment groups (by fruiting stage).

Effect of elevated CO_2 on nodulating plants under mineral N supply

With the exception of 19 and 20 DAG, average PI of nodulating plants supplied with N+ nutrient solution under elevat-

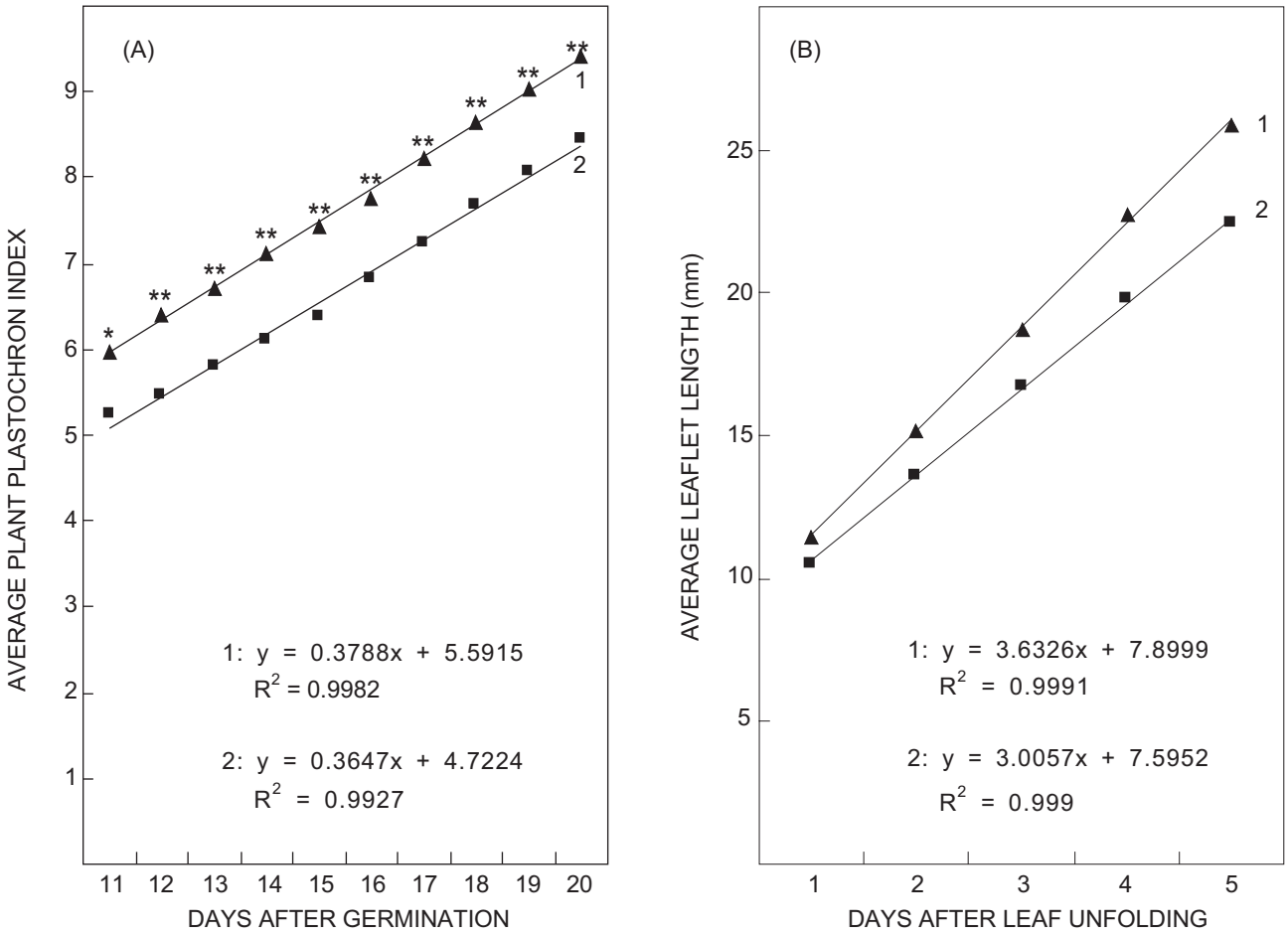

Figure 1: Average plant plastochron index (A) and length of leaflets on node 6 (B) of nodulating plants supplied with N+ nutrient solution under elevated (REn, 1) and ambient (RAn, 2) CO_2. Regression equations for the lines are embedded with the respective figures. Values at the data points of the line 1 with * or ** are significantly higher than those of the line 2 at the same or closest X value at P = 0.01 or P = 0.001 respectively

ed CO_2 (REN) was significantly higher (P = 0.05) than that of plants under ambient CO_2 (RAN) throughout the experimental period (Figure 2A). The average plastochron duration was 2.30 ± 0.07 days under elevated CO_2 (REN) and 2.34 ± 0.07 under ambient CO_2 (RAN), but like in nodulating plants treated with N-free nutrient solution, the values were not significantly different at P = 0.05. A boost in plant growth in terms of PI at short-term exposure to CO_2 is also evident. However, unlike the REn- and RAn-treated plants, Figure 2B shows that leaflet elongation rate (L_fER) was higher (P = 0.05) in REN-treated plants (5.70 ± 0.27) than in RAN-treated plants (4.67 ± 018) and the values were significantly different at P = 0.05. This indicates therefore, that L_fER is enhanced by elevated CO_2 under high nitrogen supply (fixed nitrogen and mineral nitrogen).

Effect of elevated CO_2 on non-nodulating plants under mineral N supply

The average PI of non-nodulating plants supplied with mineral N under elevated CO_2 (rEN) and ambient CO_2 (rAN) is shown in Figure 3A. The plastochron duration in rEN-treated plants was significantly higher at P = 0.05 (2.03 ± 0.05 days) than that calculated for rAN plants under (2.21 ± 0.06 days). PI values throughout the experimental period were significantly higher at P = 0.001 in rEN-treated plants than in rAN-treated plants. Similarly, the leaflets elongation rate (Figure 3B) was significantly higher at P = 0.01 in rEN-treated plants (5.87 ± 0.18) than in rAN-treated plants (4.72 ± 0.36). The data indicate that both short-term and long-term exposure to elevated CO_2 enhanced leaf growth in non-nodulating *P. sativum* L. plants, which were supplemented with mineral N.

Interactive effect of elevated CO_2, nodulation and mineral N supply on rate of leaf appearance (RLA)

The average plastochron indices of all plants grown under elevated CO_2 were significantly higher than that of their counterpart grown under ambient CO_2 throughout the period of analysis. An example is shown of nodulating plants supplied with mineral N in Figure 1. The average PI of nodulating plants supplied with N+ nutrient solution under elevated CO_2 (REN) was significantly higher (P = 0.05) than that of plants under ambient CO_2 (RAN) throughout the experimental period, except for 19 and 20 DAG.

Table 1 shows a matrix comparison (horizontal against vertical) of the rate of leaf appearance (plastochron duration) in plants under all treatments. A comparison of RAn- and RAN-treated plants shows that the supply of mineral N to nodulating plants under ambient CO_2 increased RLA

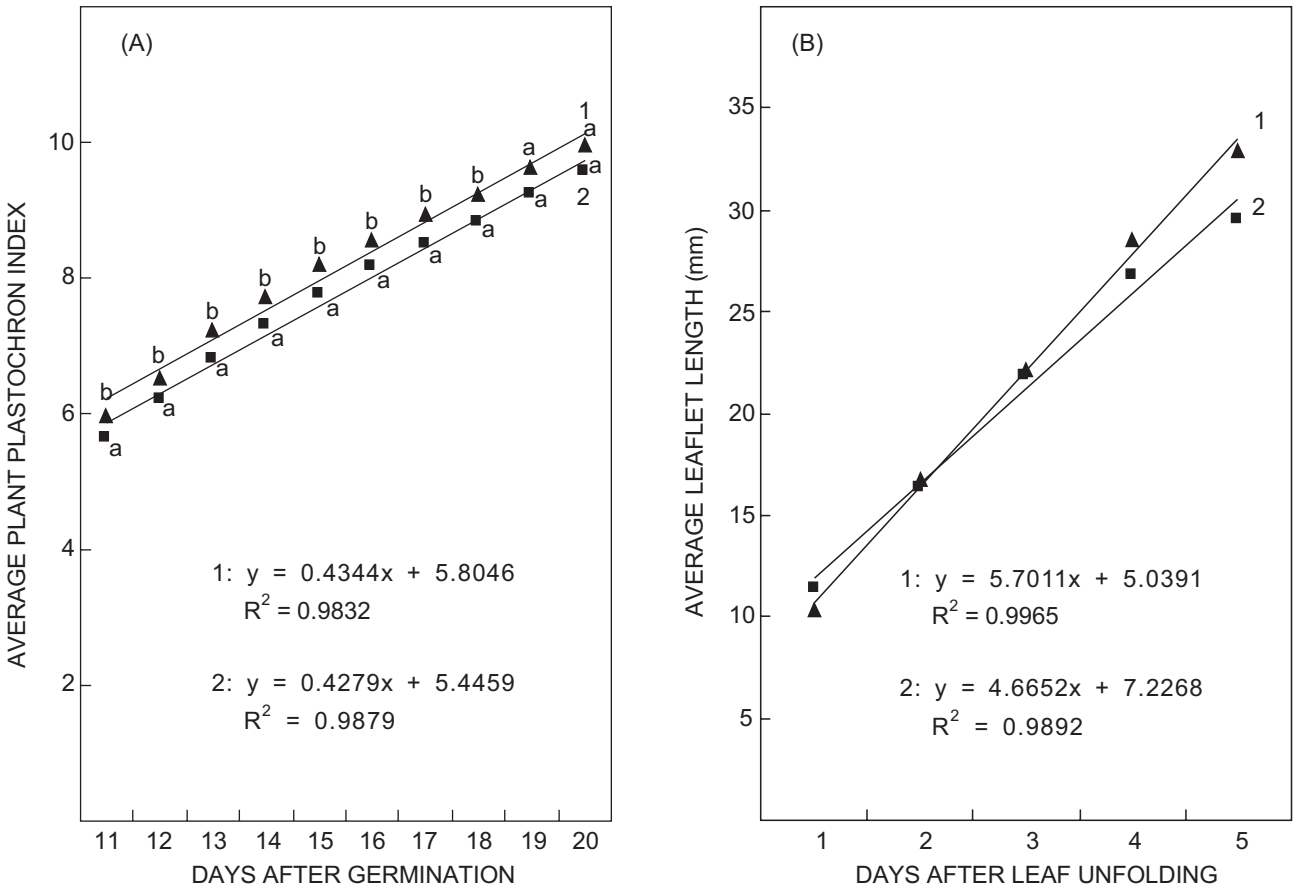

Figure 2: Average plant plastochron index (A) and length of leaflets on node 6 (B) of nodulating plants supplied with N+ nutrient solution under elevated (REN,1) and ambient (RAN, 2) CO_2. Regression equations for the lines are embedded with the respective figures. Values on same X point bearing same letter are not significantly different at P = 0.05

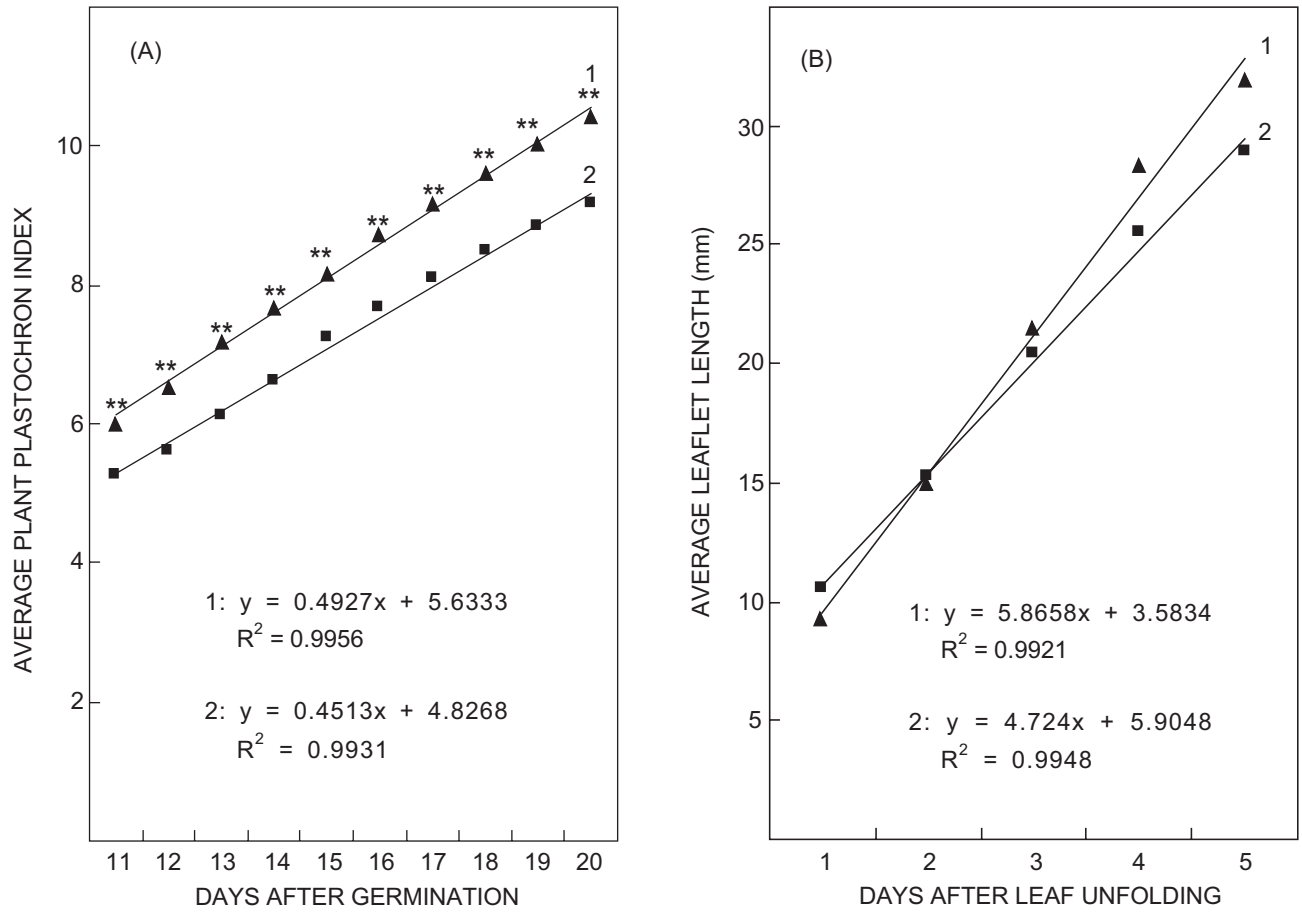

Figure 3: Average plant plastochron index (A) and length of leaflets on node 6 (B) of non-nodulating plants supplied with N+ nutrient solution under elevated (rEN,1) and ambient (rAN, 2) CO_2. Regression equations for the lines are embedded with the respective figures. Values at the data points of the line 1 with ** are significantly higher than those of the line 2 at the same or closest X value at P = 0.001

Table 1: Matrix comparison of differences between plastochron duration (rate of leaf appearance, RLA) of *P. sativum* under all treatments: nodulating plants supplied with N-free nutrient solution under elevated (REN) and ambient (RAN) CO_2, nodulating plants supplied with N+ nutrients solution under elevated (REN)and ambient (RAN) CO_2; and non-nodulating plants supplied with N+ nutrient solution under elevated (rEN) and ambient (rAN) CO_2

	Nodulating				Non-nodulating	
	No mineral N supply		With mineral N supply			
	Elevated (REn)	Ambient (RAn)	Elevated (REN)	Ambient (RAN)	Elevated (rEN)	Ambient (rAN)
REn						
RAn						
REN		b				
RAN	b	b				
rEN	**	**	*	*		
rAN	b	b			b	

Blank cells show no significant difference, b indicates significant difference at P = 0.05, * indicates significant difference at P = 0.01, ** indicates significant difference at P = 0.001

(plastochron duration) significantly at P = 0.05. However, significant induction of RLA due to mineral N supply to nodulating plants (REn versus REN) did not take place under elevated CO_2. Elevated CO_2 also had no effect on the RLA of nodulated plants (compare RAn to REn, RAN to REN). RLA in plants grown under ambient CO_2 was significantly higher in plants in which N was supplemented (rAN), than under fixed N alone (RAn), however, there was no significant difference between nodulated and non-nodulated plants, when nodulated plants were supplied with additional mineral N

(RAN versus rAN). However, elevated CO_2 induced RLA significantly in non-nodulated plants above that in nodulated plants which were grown with or without mineral N supplement (REn and REN vs rEN).

With RAN serving as control, Figure 4 further illustrates the interactive effects of elevated CO_2, nodulation and mineral N on RLA, based on the percentage increase in RLA in other treatments, compared to RAn-grown plants. The increase in RLA under ambient CO_2 due to mineral N supply in both nodulating (RAN, 17%) and non-nodulating (rAN, 24%) plants respectively, was significant at P = 0.05 (Table 1). Increase in RLA (4%) due to elevated CO_2 was not significant

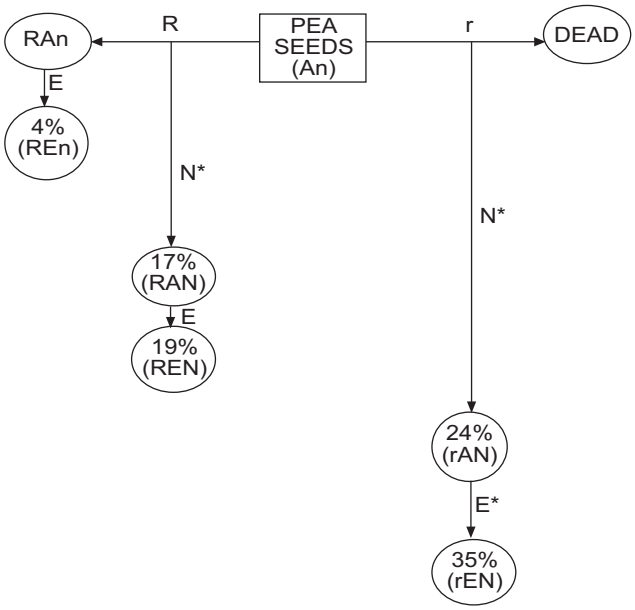

Figure 4: Summary of the interactive effects of nodulation, elevated CO_2 and N status on the rate of leaf appearance (RLA, plastochron duration) in plants under the different treatment conditions compared to that of nodulating plants under ambient CO_2 without N supply (RAn). * shows that the treatment had a significant effect. (R — nodulation/Rhizobium, r — absence of nodulation, N — mineral N supply, n — no mineral N supply, A — Ambient CO_2, E — elevated CO_2)

in nodulating plants without mineral N supply (REn). The further increase in RLA (2%) in nodulating plants above that already effected by mineral N supply (REN) was also not significant. Figure 4 therefore illustrates that RLA was only significantly affected by mineral N supply in nodulating plants. Furthermore, the effect of elevated CO_2 was only significant in non-nodulating plants supplied with mineral N (rEN).

Interactive effect of elevated CO_2, nodulation and mineral N supply on leaflet elongation rate (L_fER)

The matrix comparison (horizontal against vertical) of the leaflet elongation rate (L_fER) between treatments on Table 2 shows some interesting results. Mineral N enhanced the L_fER of nodulated plants irrespective of CO_2 condition (compare RAn to RAN and REn to REN). Elevated CO_2 only enhanced L_fER in nodulated plants in the presence of mineral N supply. L_fER was significantly higher in non-nodulated than in nodulated plants (RAn against rAN). The difference becomes insignificant with the addition of mineral N to nodulating plants (RAN compared to rAN). Similar results were obtained under elevated CO_2 (compare REn to REN, and both to rEN).

The interactive effects of elevated CO_2, nodulation and mineral N on L_fER, based on the percentage increase in RLA in other treatments compared to RAn is further illustrated in Figure 5. Mineral N supply increased L_fER significantly in both nodulating (RAN, 55%) and non-nodulating (rAN, 57%) plants. However, the effect of elevated CO_2 on L_fER was only significant where mineral N was supplied to nodulating plants (REN, 90%) and even more significant in non-nodulating plants supplied with mineral N (rEN, 97%).

Discussion

C_3 species are known to be able to utilize elevated [CO_2] to support faster growth, especially during the early stages of growth (Murray 1995). The insignificant differences in plastochron duration values of plants grown under elevated CO_2 from 11 DAG show that short-term exposure to elevated CO_2 enhanced the growth of *Pisum sativum* plants by inducing an enhanced plastochron index (PI) rate. However under elevated CO_2 conditions, enhanced plant growth occurred

Table 2: Matrix comparison of differences between leaflet elongation rate (L_fER) of *P. sativum* under all treatments: nodulating plants supplied with N-free nutrient solution under elevated (REN) and ambient (REN) CO_2; nodulating plants supplied with N+ nutrient solution under elevated (REN) and ambient (RAN) CO_2; and non-nodulating plants supplied with N+ nutrient solution under elevated (rEN) and ambient (rAN) CO_2

	Nodulating				Non-nodulating	
	No mineral N supply		With mineral N supply			
	Elevated (REn)	Ambient (RAn)	Elevated (REN)	Ambient (RAN)	Elevated (rEN)	Ambient (rAN)
REn						
RAn						
REN	**	**				
RAN	b	**	b			
rEN	**	**		**		
rAN	b	**	b		*	

Blank cells show no significant difference; b indicates significant difference at P = 0.05, * indicates significant difference at P = 0.01, ** indicates significant difference at P = 0.001

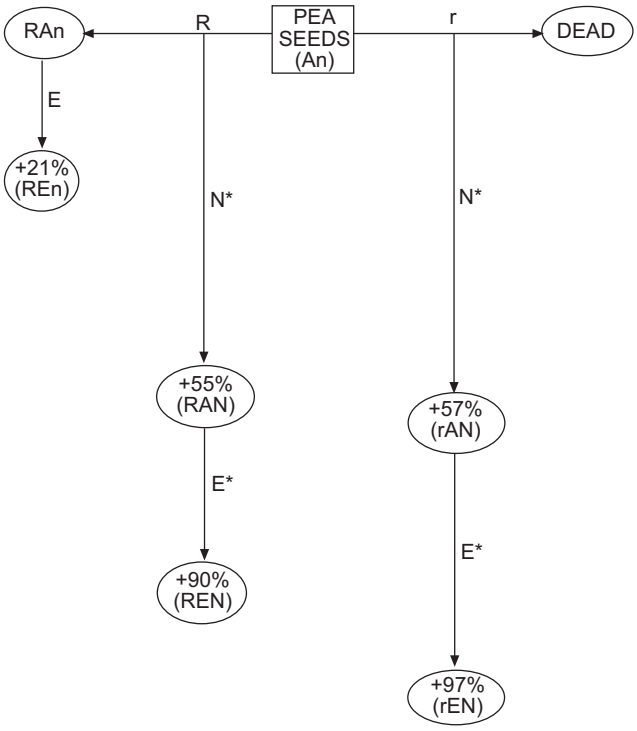

Figure 5: Summary of the interactive effects of nodulation, elevated CO_2 and N status on leaflet elongation rate (L_fER) in plants under the different treatment conditions compared to that of nodulating plants under ambient CO_2 without N supply (RAn). * shows that the treatment had a significant effect. (R — nodulation/Rhizobium, r — absence of nodulation, N — mineral N supply, n — no mineral N supply, A — Ambient CO_2, E — elevated CO_2)

only in non-nodulated plants in long-term experiment in which nitrogen was supplied.

Interactive effect of elevated CO_2, nodulation and mineral N supply on general plant growth in terms of rate of leaf appearance (RLA)

The rate of leaf appearance (RLA) was significantly increased in the absence of nodules (with mineral N supply). Mineral N supply increased RLA significantly under ambient CO_2. Elevated CO_2 only affected RLA significantly (positively) in plants which did not have nodules. Our observations thus support the contention that nutrient availability, especially nitrogen, affects RLA (Truong and Duthion 1993, Turc and Lecoeur 1997). The influence was however greatly reduced under elevated CO_2, suggesting a lack of interaction between elevated CO_2 and mineral N in the influence of RLA. Field experiments using *Lolium perenne* and *Trifolium repens* in managed grassland have established that the response of vegetative growth to elevated CO_2 in terms of nitrogen availability is nitrogen-dependent in grasses but independent in nitrogen-fixing legumes (Hebeisen *et al.* 1997, Daepp *et al.* 2000, Wagner *et al.* 2001). Proportional allocation of photosynthates is another factor, which may prevent the effect of sustained elevated CO_2 being established significantly in plants with access to both fixed nitrogen and mineral nitrogen (Kutík *et al.* 1995). Results of the experiments reported here suggest that these statements could be true for rate of leaf appearance in nodulating *P. sativum* plants.

The temperature range in all growth conditions used in the experiments was maintained at identical levels under controlled environmental conditions. If RLA is solely dependent on temperature as suggested by Turc and Lecoeur (1997), its value should not be significantly different in plants under the same CO_2 conditions. However, the significant increase in RLA in non-nodulated plants under elevated CO_2, points to a possibility of an increase in the production of photosynthates which, unlike in the case of nodulated plants where assimilates are being shared by the plant as well as by the nodules, the non-nodulated plants can make full use of these resources. As a result non-nodulated plants have a larger carbohydrate pool available to boost RLA compared with nodulated plants grown under elevated CO_2 conditions. Our results support those of Truong and Duthion (1993), which showed that dry matter accounts for variability in RLA in peas under same temperature conditions. Interestingly, an increase in photosynthesis in non-nodulating *P. sativum* grown under 1 000µmol mol^{-1} and high N supply has been reported by Riviere-Rolland *et al.* (1996). Philips *et al.* (1976) showed that long-term exposure to very high (1 200µmol mol^{-1}) CO_2 with additional mineral nitrogen supply resulted in vigorous, balanced growth of pea plants with no detrimental effect on nitrogenase activity. A major flaw in the experimental procedure was that Philips *et al.* (1976) observations were based on 6h per day exposure of peas to elevated CO_2 — this was hardly 'continuous' exposure! Continuous exposure to elevated CO_2 as in the case of the experiments we currently report in this paper, possibly inhibited nodule activity completely in nodulated plants which were supplied continuously with mineral N, thus fixed nitrogen perhaps did not contribute positively to the growth of the leaves of plants under elevated CO_2.

Effect of elevated CO_2, nodulation and mineral N supply on leaflet elongation rate (L_fER)

It is clear that high growth rate under high nutrient availability, promotes a strong response to elevated CO_2 (Daepp *et al.* 2001). In addition various experiments have demonstrated that the strong growth effects of elevated CO_2 on legumes, relative to effects seen in non-legume species, are obtained only in the presence of high mineral nutrient supply (Nijs *et al.* 1989, Körner 1995, Pritchard *et al.* 1997). In the experiments reported here, elevated CO_2 enhanced L_fER significantly in plants supplied with mineral N, irrespective of the presence or absence of nodules. L_fER was enhanced significantly by mineral N supply in nodulating plants under both ambient and elevated CO_2 conditions. Clearly, absence or presence of nodules does not appear to affect L_fER in plants supplied with mineral N.

The experimental evidence reported here shows that CO_2 levels as high as 1 000µmol mol^{-1} may not significantly affect the growth of nodulating *Pisum sativum* L. However, the plant has an opportunity to enhance its canopy, only when soil N is adequate but would benefit more from future elevation in CO_2 in the absence of nodules and with either an adequate soil (N), or through application of additional nitrogen.

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